EPA Reviewer: Monique M. Perron, Sc.D **Signature**: _____
Risk Assessment Branch I, Health Effects Division (7509P) **Date:** _____
EPA Secondary Reviewer: Connor Williams, MHS **Signature**: _____
Risk Assessment Branch I, Health Effects Division (7509P) **Date:** _____

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DATA EVALUATION RECORD[1]

STUDY TYPE: Non-guideline mechanistic study

PC CODE: 129210 **DP BARCODE:** D432127

TEST MATERIAL (PURITY): Triflumezopyrim (99.4% a.i.)

SYNONYMS: DPX RAB55, 2,4-Dioxo-1-(5-pyrimidinylmethyl)-3-(3-(trifluoromethyl)-phenyl)-2H-pyrido(1,2-a)pyrimidinium inner salt

CITATION: Mukerji, P. (2015); Triflumezopyrim (DPX-RAB55) technical: liver mechanistic study in male mice. DuPont Haskell Laboratory, Newark, Delaware, USA. Testing Facility Report No.: DuPont-44649. December 4, 2015. MRID 49382234.

SPONSOR: E.I. du Pont de Nemours and Company, Wilmington, Delaware 19898

EXECUTIVE SUMMARY:

The objective of this study was to evaluate liver mechanistic parameters (microscopic pathology, cell proliferation, and induction of cytochrome P450 enzyme activities and gene expression) in male mice following exposure to triflumezopyrim. Triflumezopyrim was administered to male Crl:CD-1 (ICR) mice (thirty animals/concentration) at concentrations of 0, 200, 800, 2500, and 7000 ppm, or 1000 ppm of a positive control substance, phenobarbital salt. Ten male mice per group were sacrificed at each time point on test days 3, 8, and 29 (equivalent to 2, 7, or 28 days of dietary exposure, respectively, due to initiation of exposure on test day 1). The overall mean daily intake of triflumezopyrim at 200, 800, 2500, and 7000 ppm was calculated as 35, 149, 420, and 1210 mg/kg/day, respectively, for the day 3 subset; 35, 151, 450, and 1275 mg/kg/day, respectively, for the day 8 subset; and 32, 133, 411, and 1273 mg/kg/day, respectively, for the day 29 subset. Parameters evaluated during the in-life phase included body weight, body weight gain, food consumption, and clinical signs. Liver sections taken from all animals at necropsy were examined for microscopic pathology (using hematoxylin and eosin staining), cell proliferation (Ki67 labelling), and induction of cytochrome P450 enzyme activities. Gene expression for cytochrome P450 enzymes was evaluated in livers from the control, 7000 ppm triflumezopyrim, and positive control groups on Day 3 and Day 8.

Under the conditions of this study, cell proliferation was increased by approximately 2-fold compared to controls on Day 8 at 7000 ppm. There were no statistically significant increases at doses ≤2500 ppm. The number of labeled hepatocytes/mm^2 returned to normal by Day 29. The proliferation response with the positive control was more pronounced (15-fold increase by

[1] This DER was generated by modifying the study summary in a Tier II document (MRID 49382105).

Day 3) and was still present at Day 29 (2-fold increase). CYP2B10 expression (an indicator of constitutive androstane receptor (CAR) activation) was increased 29.7-fold and 99-fold following administration of 7000 ppm for 3- and 8-days of exposure, respectively. This response was not as potent as the positive control (phenobarbital), which increased CYP2B10 expression by 100-fold and 247-fold following administration for 3- and 8-days of exposure. Both triflumezopyrim and the positive control produced slight activation of PXR as assessed by expression of CYP3A11 (approximately 3-fold increase by Day 8 for both).

CYP2B enzyme activity was also increased. Changes in activities were only present at one or more time points at the 2500 and 7000 ppm and not observed at 200 or 800 ppm. At 7000 ppm, CYP2B enzyme activity was increased 2.6-, 2.7-, and 2.0-fold above the negative control (all statistically significant) on Days 3, 8, and 29, respectively. At 7000 ppm, increases in CYP1A enzyme activity and CYP1A1 gene expression similar to phenobarbital were also observed. Total P450 was statistically significantly increased only on test day 8 in the 7000 ppm (2.4-fold induction) compared to the control group. Thus, the elevation in total P450 in this group was similar, although slightly lower than that seen with phenobarbital.

This non-guideline mechanistic study is adequate for the mode of action evaluation for liver tumors observed in male mice in the mouse carcinogenicity study (MRID 49382174).

COMPLIANCE: Signed and dated GLP, Quality Assurance, and Data Confidentiality statements were provided.

I. MATERIALS AND METHODS

A. MATERIALS

1. Test material: Triflumezopyrim technical
   Lot/Batch #: RAB055-037
   Purity: 99.4%
   Description: Solid
   CAS #: 1263133-33-0
   Stability of test compound: Analyses confirmed that test material was stable in feed for at least 17 days at room temperature, was distributed uniformly in the feed, and was present in the feed at targeted concentrations.
2. Vehicle and/or control: Untreated diet
   Positive control: Phenobarbital (91.35% by analysis)
   Alzet® osmotic pump: 100 µL capacity, filled with 5-Bromo-2-Deoxyuridine (BrdU; 40 mg/mL) sterile solution in 0.5 N sodium bicarbonate buffer.
3. Test animals
   Species: Mouse
   Strain: Crl:CD-1(ICR)
   Age at initial dosing: Approximately 7 weeks old
   Weight at initial dosing: 28.3–31.4 g
   Source: Charles River Laboratories, Inc., Raleigh, North Carolina, USA
   Acclimation period: At least 5 days
   Diet: PMI® Nutrition International, LLC Certified Rodent LabDiet® (#5002), *ad libitum*. During the test period, test substance was incorporated into the feed of all animals except controls.
   Water: Tap water, *ad libitum*
   Housing: Animals were housed singly in solid-bottom caging with bedding mixed with enrichment
4. Environmental conditions
   Temperature: 20–26°C
   Humidity: 30–70%
   Air changes: Not reported
   Photoperiod: Alternating 12-hour light and dark cycles

B. STUDY DESIGN

1. In-life initiated/completed
   26-June-2015 to 24-July-2015

2. Animal assignment and treatment

   The study design is summarized in Table 1. Six groups of 30 young adult male animals/concentration were administered concentrations of 200, 800, 2500, or 7000 ppm of triflumezopyrim, or 1000 ppm of a positive control substance, phenobarbital. The male animals received 35, 149, 420, and 1210 mg/kg/day, respectively, for the Day 3 subset; 35, 151, 450, and 1275 mg/kg/day, respectively, for the Day 8 subset; and 32, 133, 411, and 1273 mg/kg/day, respectively, for the Day 29 subset. The dietary concentrations for this study were selected based on effects observed at concentrations evaluated in previous studies. The dietary route of administration was

selected because it is consistent with the route of exposure in previous studies with the test substance. Animals were assigned to dose groups by computerized, stratified randomization so that there were no statistically significant differences among group body weight means. The weight variation of selected animals did not exceed ± 20% of the mean weight for each sex. Dietary administration of triflumezopyrim began on test day 1, when the animals were approximately 7 weeks old. Animal housing and husbandry were in accordance with the provisions of the *Guide to the Care and Use of Laboratory Animals* (NRC 2011).

3. Diet preparation and analysis

The test substance was added to the rodent diet and thoroughly mixed for a period of time that was adequate to ensure homogeneous distribution in the diet. The diet preparation was corrected for the sponsor-reported purity. Control diets were mixed for the same period of time. All diets were prepared and stored under the conditions of established triflumezopyrim stability until used. The homogeneity and concentration of triflumezopyrim in the dietary mixtures was checked by analysis using ultra high-performance liquid chromatography (UHPLC) with ultraviolet (UV) detection at the beginning of the study. The diet analysis results showed that triflumezopyrim was homogeneously mixed at the targeted concentrations for all dietary concentrations. Stability of triflumezopyrim in the diet was established in previously conducted studies. Based on this information, it can be concluded that the animals received the targeted dietary concentrations of test substance during the study.

Table 1. Study design

Males									
	Day 3 Subset			Day 8 Subset			Day 29 Subset		
Group no.	No./ group	Conc. in diet (ppm)[a]	Mean daily intakes mg/kg bw	No./ group	Conc. in diet (ppm)[a]	Mean daily intakes mg/kg bw	No./ group	Conc. in diet (ppm)[a]	Mean daily intakes mg/kg bw
1	10	0 (control)	0 (control)	10	0 (control)	0 (control)	10	0 (control)	0 (control)
2	10	200	35	10	200	35	10	200	32
3	10	800	149	10	800	151	10	800	133
4	10	2500	420	10	2500	450	10	2500	411
5	10	7000	1210		7000	1275	10	7000	1273
6	10	1000 (phenobarbital)	182 (phenobarbital)	10	1000 (phenobarbital)	178 (phenobarbital)	10	1000 (phenobarbital)	168 (phenobarbital)

[a] Weight/weight concentration of test substance

4. Statistics

Table 2. Statistics

Parameter	Preliminary Test	Method of Statistical Analysis	
		If preliminary test is not significant	If preliminary test is significant
Body Weight Body Weight Gain Food Consumption Organ Weight Cell Proliferation Enzyme Activities	Levene's test for homogeneity and Shapiro-Wilk test for normality	One-way analysis of variance followed by Dunnett's test	Transforms of the data to achieve normality and variance homogeneity were used. The order of transforms attempted was log, square-root, and rank-order. If the log and square-root transforms failed, the rank-order was used.
Gene Expression	None	One-way analysis of variance followed by Tukey test.	

C. METHODS

1. Observations

Animals were observed at least once daily for mortality and morbidity and for signs of abnormal behaviour and appearance. An additional cage-site evaluation was conducted daily sometime between 6 a.m. and 12 p.m. to detect acute clinical signs of systemic toxicity, except on the days when careful clinical observations were conducted. On days when they were weighed, each animal was individually handled, examined for abnormal behaviour and appearance, and subjected to detailed clinical observations.

2. Body weights

All animals were weighed once per week and on the day of sacrifice.

3. Food consumption and daily intake

Food consumption was recorded for each animal over the weighing interval. From these measurements, mean daily food consumption was determined for each interval. From the food consumption and body weight data, the mean daily intake of the test substance was calculated.

4. Sacrifice and pathology

Animals were sacrificed by isoflurane anaesthesia and exsanguination on Day 3, 8, or 29. The order of sacrifice was stratified across groups. Liver and duodenum were collected from all animals at scheduled sacrifice. Tissues were saved in 10% neutral buffered formalin for histopathology (livers only) and cell proliferation evaluation (both tissues). All animals had liver sections examined microscopically for both histopathological and cellular proliferation assessment. Liver sections examined histopathologically were graded on a 4-point scale representing changes that were considered minimal (grade 1), mild (grade 2), moderate (grade 3), or severe (grade 4).

5. Cell proliferation evaluation

Animals were subcutaneously implanted with Alzet® Osmotic Pumps containing a sterile BrdU solution approximately 2 days prior to the Day 3 sacrifice or 1 week prior to the Day 8 and Day 29 sacrifice. The delivery rate for the BrdU solution corresponded to 20 μg/hr (BrdU). Implantation was conducted while animals were under isoflurane anaesthesia, with buprenorphine analgesia administered SC at the time of implantation. Liver and duodenum tissues were processed to slides and stained with anti-Ki67 substrate and then counterstained with haematoxylin to determine nuclear expression of Ki67 in hepatocytes. Slides were scanned to high resolution images, which were analysed for the number of Ki67 positive hepatocyte nuclei per square millimeter of liver tissue, using automated image analysis software. The duodenum served as a qualitative positive control for Ki67 staining. Initially, slides designated for cellular proliferation analysis were stained with anti-BrdU antibody, 3,3'-diaminobenzidine (DAB) and hematoxylin. However, during the course of scoring slides for cell proliferation, it was determined that BrdU immunohistochemical staining was inadequate. The BrdU-stained slides were maintained but were not used in the final assessment of cell proliferation. Tissue sections were therefore recut from the paraffin blocks, and processed as described above for Ki67 staining and cell proliferation evaluation.

6. Biochemical evaluation

Microsome preparation

Liver samples were stored at approximately -60ºC to -80ºC until processed to microsomes. Hepatic microsomes were prepared using differential centrifugation.

Protein analysis

The BCA protein assay was used to determine the protein concentrations for all enzyme activity assays except the total P450 which utilized the Bradford method. The final protein concentration was used to calculate the specific enzyme activity.

CYP1A (7-ethoxyresorufin O-deethylase, EROD) enzyme activity

The activity of CYP1A was assessed using ethoxyresorufin (ER) as the probe substrate. Microsome samples from untreated and treated mice were diluted to approximately 0.1 mg/mL protein concentration prior to the addition of the substrate and cofactors. Fluorescence intensities of resorufin production (EX=530 nm, EM=580 nm) were monitored on a SpectraMax i3 plate reader (Molecular Devices, Sunnyvale, California, U.S.A.). The change in fluorescence intensities from time 0 to 5 minutes in diluted microsomes was compared to a set of resorufin standards and CYP1A activity was expressed as pmol resorufin/min/mg of microsomal protein.

CYP2B (7-pentoxyresorufin O-dealkylase, PROD) enzyme activity

The activity of CYP2B was assessed by the same procedure as for CYP1A except the microsomes were diluted to approximately 0.2 mg/mL protein concentration and pentoxyresorufin (PR) was used as the probe substrate. The fluorescence intensities from the diluted microsomes were compared to a set of resorufin standards and CYP2B activity was expressed as pmol resorufin/min/mg of microsomal protein.

CYP3A (testosterone 6-β hydroxylase) enzyme activity

The activity of CYP3A was assessed using testosterone as a probe substrate. Hydroxylation of testosterone at the 6β position is a specific marker for CYP3A activity. The final microsomal protein concentration target was approximately 2.0 mg/mL. After 5 minutes of incubation, the reaction was terminated by removing a 200 µL aliquot into 800 µL of internal standard (250 ppb corticosterone in methanol). Sample extracts were analyzed by LC/MS/MS for 6β-hydroxytestosterone. The activity of CYP3A was expressed as pmol 6β-hydroxytestosterone/min/mg of microsomal protein.

CYP4A (lauric acid ω-hydroxylase) enzyme activity

The activity of CYP4A was assessed using lauric acid as a probe substrate. The quantity of the specific product of CYP4A oxidation, 12-hydroxydodecanoic acid was measured by LC/MS/MS method. The final protein concentration was approximately 1.0 mg/mL. The reaction was initiated by the addition of 5 µL of 20 mM lauric acid and incubated at 37°C for approximately 5 minutes. After 5 minutes of incubation, the reaction was terminated by removing a 250 µL aliquot into 750 µL of acetonitrile. The sample extracts were analyzed for 12-hydroxydodecanoic acid by LC/MS/MS. The activity of CYP4A was expressed as pmol 12-hydroxydodecanoic acid/min/mg of microsomal protein.

Total cytochrome P450 enzyme activity

The hepatic microsomal suspensions were analyzed for total cytochrome P450 levels. The total microsomal cytochrome P450 contents were measured by spectral analysis according to the method of Suk-Jung Choi *et al.* with some modifications. Microsomes from untreated and treated mice were diluted to approximately 1.0 mg/mL. An aliquot of each sample was transferred to 2 separate 96 well plates in duplicate. To each well, 10 µL 0.5 M sodium hydrosulfite solution was added. The control plate was immediately placed into a UV plate reader. The spectra (450 and 490 nm) were recorded at room temperature for 6 minutes at 1 minute intervals. The second plate was placed in a sealed chamber and exposed to CO in a sealed chamber for 3 minutes. After incubation, the CO exposed plate was transferred to the UV plate reader. The spectra (450 and 490 nm) were recorded at room temperature for 6 minutes at 1 minute intervals. Total P450 concentration for each microsome sample according to the following formula:

$[P450]\ (nM) = (\Delta A_{PC} - \Delta A_{P})/0.091*pathlength*protein,\ mg/mL$

Where:

ΔA_{PC} = average absorbance difference of the samples in the CO exposed plate (PC) (abs 450 – abs 490)

ΔA_{P} = average absorbance difference of the samples in the unexposed plate (P) (abs 450 – abs 490)

0.091 = micromolar difference extinction coefficient

Pathlength = distance from the bottom of the plate to the top of the sample.

Analyses of cytochrome P450 gene expression

Hepatic cytochrome P450 gene expression was assessed for control, 7000 ppm and phenobarbital (1000 ppm) dosed animals collected on Day 3 and 8. Liver tissue was processed to RNA which was reverse transcribed to cDNA, and quantitative real-time PCR was performed. Gene expression analysis with Taqman primers specific to CYP 1A1, 2B10, 3A11, 4A10 was completed using the housekeeping gene β-Actin for normalization. Fold-change in cytochrome P450 isozyme gene expression was expressed relative to the average of vehicle control treated animals.

II. RESULTS AND DISCUSSION

A. OBSERVATIONS

1. Clinical signs of toxicity

No toxicologically significant clinical signs were observed for any dietary concentration in any animal.

2. Mortality

No instances of mortality occurred during the course of this study.

B. BODY WEIGHT AND BODY WEIGHT GAIN

There were no toxicologically significant effects on body weights or body weight gains (Tables 3 and 4).

C. FOOD CONSUMPTION AND FOOD EFFICIENCY

There were no toxicologically significant effects on food consumption or food efficiency (Table 5).

Table 3. Body weights (g)

Day	0 ppm Control	200 ppm Triflumezopyrim	800 ppm Triflumezopyrim	2500 ppm Triflumezopyrim	7000 ppm Triflumezopyrim	1000 ppm Phenobarbital
Day 3 Subset						
Day 1	30.2	30.3	30.5	30.5	29.6	30.1
Day 3	31.6	31.7	31.9	31.7	29.9	31.7
Day 8 Subset						
Day 1	30.5	29.7	30.2	29.5	31.4	30.3
Day 8	32.6	29.6	32.1	31.6	33.3	33.8
Day 29 Subset						
Day 1	28.5	28.3	28.5	28.8	28.3	28.4
Day 29	35.3	35.5	34.5	35.0	34.5	36.9

Table 4. Mean body weight gain (g)

Parameter	0 ppm Control	200 ppm Triflumezopyrim	800 ppm Triflumezopyrim	2500 ppm Triflumezopyrim	7000 ppm Triflumezopyrim	1000 ppm Phenobarbital
Day 3 Subset						
Body weight gain, Day 1–3 (% control)	1.4 (control)	1.4 (2.2)	1.4 (5.1)	1.2 (-13.1)	0.3[a] (-80.3)	1.6 (16.8)
Day 8 Subset						
Body weight gain, Day 1–8 (% control)	2.1 (control)	-0.1 (-105.2)	1.9 (-11.4)	2.1 (-1.0)	2.0 (-6.2)	3.4[b] (62.9)
Day 29 Subset						
Body weight gain, Day 1–29 (% control)	6.8 (control)	7.2 (6.0)	6.1 (-10.9)	6.2 (-9.0)	6.2 (-9.4)	8.4 (24.1)

[a] Significantly different from control by Dunnett 2 Sided test, $p < 0.05$.
[b] Significantly different from control by Dunnett Non-Parametric 2 Sided test, $p < 0.05$.

Table 5. Mean food consumption (g/animal/day)

Parameter	0 ppm Control	200 ppm Triflumezopyrim	800 ppm Triflumezopyrim	2500 ppm Triflumezopyrim	7000 ppm Triflumezopyrim	1000 ppm Phenobarbital
Day 3 Subset						
Food consumption, Day 1–3 (% control)	5.2 (Control)	5.5 (6.0)	5.8[a] (12.3)	5.2 (1.4)	5.1 (-0.4)	5.6 (8.6)
Day 8 Subset						
Food consumption, Day 1–8 (% control)	5.5 (control)	5.2 (-5.2)	5.8 (5.4)	5.4 (-1.8)	5.8 (4.3)	5.6 (1.4)
Day 29 Subset						
Food consumption, Day 1–29 (% control)	5.4 (Control)	5.2 (-5.0)	5.3 (-2.7)	5.3 (-2.3)	5.7 (4.9)	5.7 (4.6)

[a] Significantly different from control by Dunnett Non-Parametric 2 Sided test, $p < 0.05$.

D. SACRIFICE AND PATHOLOGY

1. Gross pathology, organ weights, and histopathology

Test substance-related effects included increased liver weights and microscopic findings of minimal hepatocellular centrilobular hypertrophy at test Days 3, 8 and 29 in mice administered ≥2500 ppm of the test substance (Table 6). No test substance-related microscopic findings were observed at any time point in mice administered 800 ppm of test substance or less. No other test substance-related microscopic findings were observed. Effects of the positive control, 1000 ppm phenobarbital, included increased liver weights and microscopic findings of minimal to moderate hepatocellular centrilobular hypertrophy at test Days 3, 8 and 29. Additional effects of the positive substance included a minimal to moderate increase in hepatocellular mitotic figures at Day 3, a minimal to mild increase in hepatocellular mitotic figures at Day 8, minimally increased hepatocellular mitotic figures at Day 29 and an increase in individual cell necrosis of hepatocytes at Day 29. All other microscopic findings in negative control, positive control, and test substance-exposed groups were consistent with normal background lesions in mice of this age and strain.

2. Cellular proliferation

Ki67 is a nuclear protein expressed during cellular proliferation and absent from resting cells. Thus, only actively proliferating cells, such as normal duodenal crypt epithelium, will stain positively with immunohistochemistry for anti-Ki67 antibody. Cells staining positively have a distinct, slightly granular, medium brown nucleus.

At 7000 ppm, cell proliferation was increased by approximately 2-fold compared to controls on Day 8 (Table 6). There were no statistically significant increases at doses ≤2500 ppm. The number of labeled hepatocytes /mm^2 returned to normal by Day 29, which is consistent with many other CAR-activating chemicals in mice. The proliferation response with the positive control was more pronounced (15-fold increase by Day 3) and was still present at Day 29 (2-fold increase).

The cellular proliferation data are presented as “number of Ki67 positive hepatocyte nuclei per square millimetre,” which may have underestimated the magnitude of the response. Instead these data should have been presented as Labeling Indices to more accurately reflect the proliferative response. The proliferative responses observed are consistent with the induction of CYP2b expression as previously described.

Table 6. Centrilobular hepatocellular hypertrophy and hepatocellular proliferation (Ki67 labeling)

	0 ppm Control	200 ppm Triflumezopyrim	800 ppm Triflumezopyrim	2500 ppm Triflumezopyrim	7000 ppm Triflumezopyrim	1000 ppm Phenobarbital
Day 3 Subset						
Centrilobular hypertrophy[a]	0	0	0	1	2	10
minimal	-	-	-	1	2	10
Hepatocellular proliferation[b]	10.7±3.4	10.1±6.2	10.5±3.4	11.6±5.2	13.6±4.1	154.1±78.2[c]
Day 8 Subset						
Centrilobular hypertrophy	0	0	0	1	7	10
minimal	-	-	-	1	7	0
mild	-	-	-	-	-	10
Heptacellular proliferation	16.0±5.7	9.5±7.4[d]	9.4±2.6[d]	16.4±4.6[d]	32.7±19.7[d]	80.5±26.1[d]
Day 29 Subset						
Centrilobular hypertrophy	0	0	0	3	7	10
Minimal		-	-	3	7	0
Mild		-	-	-	-	1
Moderate		-	-	-	-	9
Hepatocellular proliferation	13.0±3.5	13.5±7.0	14.2±3.3	24.3±17.8	17.5±7.5	25.0±8.8[c]

[a] 10 animals per group per time point.
[b] Number of Ki67 positive hepatocyte nuclei per square milliment (mm); expressed as the mean (standard deviation)
[c] Significantly different from control by $p<0.05$, Dunnett
[d] Significantly different from control by $p<0.05$, Dunnett Non-Parametric

3. Biochemical evaluation

Hepatic enzyme activity and gene expression

Hepatic enzyme activity following triflumezopyrim and phenobarbital exposures are presented in Tables 7-9. Gene expression data are presented in Table 10.

Phenobarbital (positive control)

As expected, administration of 1000 ppm of phenobarbital, a prototypical inducer of the constitutive androstane receptor (CAR), produced increases in CYP2B enzyme activity with corresponding marked increases in hepatic CYP2B10 gene expression. CYP2B enzyme activity was statistically significant at all time points, with the highest response of 7.1-fold above the negative control (0 ppm) occurring on test Day 8. Correlative but more pronounced fold changes in hepatic CYP2B10 gene expression were present at both the 3- and 8-day evaluations where gene expression showed marked, statistically significant increases of 100.2- and 246.9-fold above the negative control, respectively.

Phenobarbital administration was also associated with elevated enzyme activity and gene expression for CYP1A/CYP1A1 and CYP3A/CYP3A10, respectively, albeit to a lesser extent than that of CYP2B/CYP2B10. CYP1A and CYP3A enzyme activity were elevated up to 4.2- and 4.6-fold (both on day 8), respectively compared to control. The low level of response for phenobarbital-induced changes in CYP1A and CYP3A, relative to that of CYP2B, was most apparent in the hepatic gene expression data. The maximum responses for gene expression were 1.5- and 2.8-fold above the negative control (both statistically significant), for CYP1A1 and CYP3A11, respectively, compared with the much greater (approximately 250-fold) elevation in CYP2B10 gene expression.

Statistically significant, but minimal (≤2.7-fold) increases in CYP4A enzyme activity were present following phenobarbital exposure at all timepoints. However, these increases were not associated with correlative increases in hepatic CYP4A10 gene expression; CYP4A10 gene expression was actually decreased in this group on Day 3. Therefore, the statistically significant increases in CYP4A enzyme activity in the phenobarbital group were not considered to be biologically meaningful based on the minimal nature of the change and the lack of correlative changes in gene expression data. Phenobarbital is not expected to be a significant mediator of CYP4A enzyme activity or CYP4A10 gene expression.

Statistically significant increases in total P450 following administration of 1000 ppm phenobarbital was limited to a 2.4-fold elevation on test Day 8.

Triflumezopyrim

Dietary exposure to 7000 ppm triflumezopyrim increased CYP2B enzyme activity 2.6-, 2.7-, and 2.0-fold above the negative control (all statistically significant) on test Days 3, 8, and 29, respectively. Corresponding marked increases in hepatic CYP2B10 gene expression were also present with increases of 29.7- and 94.4-fold above controls at Day 3 and day 8, respectively. A statistically significant increase in CYP2B enzyme activity was also present in the 2500 ppm group on Day 8, but this increase was only 1.2-fold above controls and is not considered biologically relevant. Also, activity for this enzyme

was statistically decreased in the 200 ppm group on test Days 3 and 29. Based on the direction of change and lack of dose-response, this observation was considered to be of questionable relationship to treatment and not biologically meaningful.

In the 7000 ppm group, statistically significant increases in CYP1A enzyme activity and hepatic CYP1A1 gene expression were present at all time points and in most instances, the fold increases (up to 3.7-fold for enzyme activity and 1.8 fold for gene expression) were similar to that seen with phenobarbital. Statistically significant increases in CYP1A enzyme activity were also observed in the 800 and 2500 ppm groups at all time points, but were consistently ≤2-fold above the negative control.

CYP3A enzyme activity was statistically decreased in all triflumezopyrim-treated groups at Day 3 and in the 2500 and 7000 ppm groups at the Day 8 and 29 timepoints. However, hepatic gene expression for CYP3A11 was increased (2.1-fold and 3.4-fold on Day 3 and 8, respectively) to levels similar to that seen with phenobarbital. The lack of CYP3A increase in enzyme activity, in contrast to gene expression, may be due to the method used for enzyme activity detection (testosterone 6-β hydroxylase) as this substrate is known to be efficiently, although not exclusively, catalyzed by cytochrome P450 isoenzymes from the CYP3A subfamily. Yet, as with other hepatic cytochrome P450s evaluated, effects on CYP3A at the gene expression level were similar to that seen with phenobarbital.

CYP4A enzyme activity, generally associated with a peroxisome proliferator-activated receptor mode of action, was statistically, but minimally increased in the 7000 ppm group at all time points. The fold increases (2.1–2.7) across the time points evaluated were similar to that of phenobarbital. Statistically significant increases in CYP4A were also present in the 2500 ppm group on Day 3 and in the 800 and 2500 ppm groups on Day 8; however, in all instances, these increases were less than 2-fold. Changes in hepatic CYP4A10 gene expression in the 7000 ppm triflumezopyrim group were poorly correlated with enzyme changes. For example, hepatic CYP4A10 gene expression was increased 8.6-fold above the negative control on Day 3 along with a 2.5-fold increase in CYP4A enzyme activity. However, on Day 8 a similar fold increase in enzyme activity (2.7-fold increase) was observed without a correspondence in gene expression.

Total P450 was statistically significantly increased only on test day 8 in the 7000 ppm (2.4-fold induction) compared to the control group. Thus, the elevation in total P450 in this group was similar, although slightly lower than that seen with phenobarbital.

Table 7. CYP enzyme activity, Day 3

Sex: Male		0 ppm Control	200 ppm Triflumez-opyrim	800 ppm Triflumez-opyrim	2500 ppm Triflumez-opyrim
CYP1A (pmol/min/mg)	Mean	15.414	14.756	24.524 #1	31.387 #1
	SD	1.941	2.020	4.238	6.122
	N	10	10	10	10
CYP2B (pmol/min/mg)	Mean	13.971	9.836 #1	15.423	17.194
	SD	3.061	1.604	3.579	2.583
	N	10	10	10	10
CYP3A (pmol/min/mg)	Mean	2876.938	2124.165 @2	2279.938 @2	2184.124 @2
	SD	570.308	576.726	437.163	315.614
	N	10	10	10	10
CYP4A (pmol/min/mg)	Mean	2010.222	2190.262	2754.963	3190.404 #1
	SD	336.214	593.860	912.783	769.929
	N	10	10	10	10
TP450 (nmol/mg)	Mean	324.790	257.948	330.155	376.537
	SD	59.539	38.504	70.997	83.754
	N	10	10	10	10

Sex: Male		7000 ppm Triflumez-opyrim	1000 ppm Phenobarb-ital
CYP1A (pmol/min/mg)	Mean	41.061 #1	21.088 #1
	SD	4.867	4.873
	N	10	10
CYP2B (pmol/min/mg)	Mean	35.770 #1	48.974 #1
	SD	7.256	11.146
	N	10	10
CYP3A (pmol/min/mg)	Mean	1684.193 @2	5717.135 @2
	SD	294.155	1389.879
	N	10	10
CYP4A (pmol/min/mg)	Mean	4984.850 #1	1454.924 #1
	SD	1525.503	380.716
	N	10	9
TP450 (nmol/mg)	Mean	400.236	260.354
	SD	129.759	64.950
	N	10	10

Table taken from pages 57-58 of the study report (MRID 49382234)

1 [#- Test: Dunnett 2 Sided p < 0.05]
2 [@ - Test: Dunnett Non-Parametric 2 Sided p < 0.05]

Table 8. CYP activity, Day 8

Sex: Male		0 ppm Control	200 ppm Triflumez-opyrim	800 ppm Triflumez-opyrim	2500 ppm Triflumez-opyrim
CYP1A (pmol/min/mg)	Mean	21.078	19.239	32.605 #1	39.310 #1
	SD	2.366	1.492	3.904	3.502
	N	10	10	10	10
CYP2B (pmol/min/mg)	Mean	16.051	15.515	16.141	19.368 #1
	SD	2.543	3.597	2.384	2.893
	N	10	10	10	10
CYP3A (pmol/min/mg)	Mean	2869.207	2667.916	2500.578	2127.377 #1
	SD	513.034	655.342	425.832	183.116
	N	10	10	10	10
CYP4A (pmol/min/mg)	Mean	2312.303	1979.744	2985.952 #1	3418.850 #1
	SD	478.182	297.467	725.681	992.088
	N	10	10	10	10
TP450 (nmol/mg)	Mean	311.284	222.827	333.247	360.033
	SD	105.170	79.606	127.811	109.593
	N	10	9	10	10

Sex: Male		7000 ppm Triflumez-opyrim	1000 ppm Phenobarb-ital
CYP1A (pmol/min/mg)	Mean	78.348 #1	89.195 #1
	SD	10.693	8.307
	N	10	10
CYP2B (pmol/min/mg)	Mean	48.909 #1	114.102 #1
	SD	7.435	7.778
	N	10	10
CYP3A (pmol/min/mg)	Mean	2279.783 #1	13264.769 #1
	SD	352.758	2139.287
	N	10	10
CYP4A (pmol/min/mg)	Mean	6234.094 #1	6254.776 #1
	SD	987.264	968.244
	N	10	10
TP450 (nmol/mg)	Mean	736.189 #1	1200.993 #1
	SD	204.401	392.022
	N	10	10

Table taken from pages 59- 60 of the study report (MRID 49382234)

1 [#- Test: Dunnett 2 Sided p < 0.05]

Table 9. CYP activity, Day 29

Sex: Male		0 ppm Control	200 ppm Triflumez-opyrim	800 ppm Triflumez-opyrim	2500 ppm Triflumez-opyrim
CYP1A (pmol/min/mg)	Mean	16.456	14.602	23.356 #1	24.234 #1
	SD	1.998	1.680	5.301	4.313
	N	10	10	10	10
CYP2B (pmol/min/mg)	Mean	14.339	10.465 #1	11.844	12.562
	SD	2.969	2.685	3.828	3.234
	N	10	10	10	10
CYP3A (pmol/min/mg)	Mean	2971.274	2322.416 #1	2377.999	1747.506 #1
	SD	520.419	362.563	601.837	310.508
	N	10	10	10	10
CYP4A (pmol/min/mg)	Mean	2043.322	1943.874	2569.370	2375.119
	SD	816.812	703.149	772.600	749.947
	N	10	10	10	10
TP450 (nmol/mg)	Mean	239.361	156.055	201.898	169.686
	SD	104.696	107.898	138.769	98.935
	N	10	10	10	9

Sex: Male		7000 ppm Triflumez-opyrim	1000 ppm Phenobarb-ital
CYP1A (pmol/min/mg)	Mean	43.533 #1	37.942 #1
	SD	9.367	8.679
	N	10	10
CYP2B (pmol/min/mg)	Mean	28.363 #1	58.079 #1
	SD	6.010	11.390
	N	10	10
CYP3A (pmol/min/mg)	Mean	1899.168 #1	6772.555 #1
	SD	260.679	1970.111
	N	10	10
CYP4A (pmol/min/mg)	Mean	4355.551 #1	2555.310
	SD	1049.291	959.306
	N	10	10
TP450 (nmol/mg)	Mean	352.648	300.684
	SD	188.326	189.562
	N	9	10

Table taken from pages 61-62 of the study report (MRID 49382234)

1 [#- Test: Dunnett 2 Sided p < 0.05]

Table 10. Quantitative Real-Time PCR: Fold difference (±standard deviation)

	Day 3			Day 8		
Enzyme	0 ppm	7000 ppm Triflumezopyrim	Phenobarbital (1000 ppm)	0 ppm	7000 ppm Triflumezopyrim	Phenobarbital (1000 ppm)
CYP1A1	1.0 (±0.3)	1.8 (±0.5) *	1.5 (±0.3) *	1.0 (±0.3)	1.7 (±0.4) *	1.4 (±0.2) *
CYP2B10	1.0 (±0.8)	29.7 (±7.1) *	100.2 (±11.5) *	1.0 (±0.5)	99.4 (±17.8) *	246.9 (±55.2) *
CYP3A11	1.0 (±0.3)	2.1 (±0.5) *	2.5 (±0.3) *	1.0 (±0.1)	3.4 (±0.6) *	2.8 (±0.4) *
CYP4A10	1.0 (±0.8)	8.6 (±4.5) *	0.3 (±0.2)	1.0 (±1.3)	2.7 (±2.6)	0.9 (±0.8)

* Statistically significant compared to control values by One-Way ANOVA with Tukey's test $p \leq 0.05$.

III. CONCLUSION

A. INVESTIGATOR'S CONCLUSIONS:

Under the conditions of this study, exposure of male mice to triflumezopyrim at a dietary concentration of 7000 ppm resulted in hepatocellular centrilobular hypertrophy, increased liver weights, and increased hepatocellular proliferation. These liver changes occurred in association with induction of cytochrome P450 enzyme activities and gene expression consistent with that seen with the prototypical CAR inducer phenobarbital, with the most pronounced changes occurring in CYP2B10 gene expression. These effects were usually observed at all time points evaluated (Day 3, 8, and 29, where applicable), except for the increase in hepatocellular proliferation, which was only observed on Day 8. Mice administered 2500 ppm did not exhibit any increase in hepatocellular proliferation, but did exhibit other liver effects (hepatocellular centrilobular hypertrophy, increased liver weights, and minimal induction of cytochrome P450 enzyme activities), with generally lower magnitude or lower incidence relative to the 7000 ppm group. There were no biologically relevant effects at 200 or 800 ppm.

The changes observed in the cytochrome P450 profile, along with the associated changes in the livers of male mice at 7000 ppm, support a phenobarbital-like mechanism of action for liver tumor induction observed in male mice following chronic exposure to that dietary concentration. The first key event in this mechanism is induction of CYP2B enzymes with resulting liver hypertrophy and increased liver weights. These changes are followed by the second key event of liver cell proliferation progressing to liver tumors with chronic exposure. Consistent with this mechanism, the second key event of increased liver cell proliferation was only seen at the 7000 ppm concentration in the current study, which is the only concentration that produced liver tumors in the 18-month mouse feeding study.

B. REVIEWER COMMENTS:

Under the conditions of this study, cell proliferation was increased by approximately 2-fold compared to controls on Day 8 at 7000 ppm. There were no statistically significant increases at doses ≤2500 ppm. The number of labeled hepatocytes/mm^2 returned to normal by Day 29. The proliferation response with the positive control was more pronounced (15-fold increase by Day 3) and was still present at Day 29 (2-fold increase). CYP2B10 expression (an indicator of CAR activation) was increased 29.7-fold and 99-fold following administration of 7000 ppm for 3- and 8-days of exposure, respectively. This response was not as potent as the positive control (phenobarbital), which increased CYP2B10 expression by 100-fold and 247-fold following administration for 3- and 8-days of exposure. Both triflumezopyrim and the positive control produced slight activation of PXR as assessed by expression of CYP3A11 (approximately 3-fold increase by Day 8 for both).

CYP2B enzyme activity was also increased. Changes in activities were only present at one or more time points at the 2500 and 7000 ppm and not observed at 200 or 800 ppm. At 7000 ppm, CYP2B enzyme activity was increased 2.6-, 2.7-, and 2.0-fold above the negative control (all statistically significant) on Days 3, 8, and 29, respectively. At 7000 ppm, increases in CYP1A enzyme activity and CYP1A1 gene expression similar to phenobarbital were also

observed. Total P450 was statistically significantly increased only on test day 8 in the 7000 ppm (2.4-fold induction) compared to the control group. Thus, the elevation in total P450 in this group was similar, although slightly lower than that seen with phenobarbital.

C. STUDY DEFICIENCIES:

The cellular proliferation data are presented as "number of Ki67 positive hepatocyte nuclei per square millimeter," which may have underestimated the magnitude of the response. Instead these data should have been presented as Labeling Indices to more accurately reflect the proliferative response.